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Targeted Correction of Single-Base-Pair Mutations with Adeno-Associated Virus Vectors under Nonselective Conditions

Xiaoming Liu, Ziying Yan, Meihui Luo, Roman Zak, Ziyi Li, Ryan R. Driskell, Yumao Huang, Nam Tran, and John F. Engelhardt*

Department of Anatomy and Cell Biology and the Center for Gene Therapy of Cystic Fibrosis and Other Genetic Diseases, University of Iowa College of Medicine, Iowa City, Iowa 52242

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Recombinant adeno-associated virus (rAAV) vectors possess the unique ability to introduce genetic alterations at sites of homology in genomic DNA through a mechanism thought to predominantly involve homologous recombination. We have investigated the efficiency of this approach using a mutant enhanced green fluorescent protein (eGFP) fluorescence recovery assay that facilitates detection of gene correction events in living cells under nonselective conditions. Our data demonstrate that rAAV infection can correct a mutant eGFP transgene at an efficiency of 0.1% in 293 cells, as determined by fluorescence-activated cell-sorting analysis. Gene repair was also confirmed using clonal expansion of GFP-positive cells and sequencing of the eGFP transgene. These results support previous findings demonstrating the efficacy of rAAV for gene targeting. In an effort to improve gene-targeting efficiencies, we evaluated several agents known to increase rAAV transduction (i.e., expression of an expressed gene), including genotoxic stress and proteasome inhibitors, but observed no correlation between the level of gene repair and rAAV transduction. Interestingly, however, our results demonstrated that enrichment of G_1/S-phase cells in the target population through the addition of thymidine moderately (~2-fold) increased gene correction compared to cells in other cell cycle phases, including G_0/G1, G_1, and G_2/M. These results suggest that the S phase of the cell cycle may more efficiently facilitate gene repair by rAAV. Transgenic mice expressing the mutant GFP were used to evaluate rAAV targeting efficiencies in primary fetal fibroblast and tibialis muscles. However, targeting efficiencies in primary mouse fetal fibroblasts were significantly lower (~0.006%) than in 293 cells, and no correction was seen in tibialis muscles following rAAV infection. To evaluate the molecular structures of rAAV genomes that might be responsible for gene repair, single-cell injection studies were performed with purified viral DNA in a mutant eGFP target cell line. However, the failure of direct cytoplasm- or nucleus-injected rAAV DNA to facilitate gene repair suggests that some aspect of intracellular viral processing may be required to prime recombinant viral genomes for gene repair events.

The ability to efficiently correct mutations in genomic DNA is considered the Holy Grail of gene therapy. However, it has also been one of the most difficult technologies to develop. Numerous approaches, including chimeric RNA-DNA oligonucleotides or chimeraplasts (3, 22), single-stranded oligonucleotides (49), triplex-forming oligonucleotides (49), and small-fragment homologous replacement (21), have been attempted with varying degrees of success. In contrast to gene addition approaches, in situ mutation repair leads to a permanent genetic change (if stem cells are targeted) as well as appropriately regulated expression of the corrected gene product. Although the current state-of-the-art approach for gene targeting is too inefficient for in vivo use, ex vivo targeting in hematopoetic stem cells is currently a major area of investigation since, theoretically, selected gene-repaired cells can be expanded in vivo (40).

A number of strategies have been utilized to facilitate site-specific gene correction both in vitro and in vivo. RNA-DNA oligonucleotide chimeraplasty was developed to target site-specific nucleotide base changes to homologous genomic sequences. This technique has demonstrated functional correction of episomal mutations and targeted repair of the sickle cell mutation in lymphoblasts. It has also been used in vivo to mutate the factor IX gene in hepatocytes and to target repair mutations in Duchenne muscular dystrophy skeletal muscles (4–6, 10, 39, 47). Single-strand oligonucleotide gene repair was subsequently developed and has also demonstrated potential to correct point mutations (27, 28). The triplex-forming oligonucleotides were developed from observations that a single-stranded DNA can form a stable triple helical structure at sequence-specific homopurine-rich regions of the genome. The combination of triplex-forming oligonucleotides and homologous recombination has led to the development of bifunctional oligonucleotides with the ability to promote DNA repair (9, 12). Another gene repair strategy based on homologous recombination uses a small DNA fragment and is termed small-fragment homologous replacement, which functions by replacing a few hundred base pairs within mutated areas of a gene. This strategy has been used in the functional correction of the ΔF508 mutation in the *CFTR* gene (11, 20).

Recombinant adeno-associated virus (rAAV) has attracted considerable interest as a vector for gene transfer and gene therapy due to its ability to infect multiple tissue types and its low immunogenicity. In addition to its ability to express transgenes from episomal and/or integrated viral genomes, rAAV has been reported to specifically target sites of homologous

* Corresponding author. Mailing address: Department of Anatomy and Cell Biology, University of Iowa, Room 1-111, Bowen Science Building, 51 Newton Rd., Iowa City, IA 52242-1109. Phone: (319) 335-7753. Fax: (319) 335-6581. E-mail: john-engelhardt@uiowa.edu.

DNA within chromosomal DNA to facilitate gene repair. With correction rates as high as 1%, more than 4 logs higher than with conventional methods such as transfection, the use of rAAV in site-specific gene targeting has increased (25, 26, 30, 31, 42, 43). Furthermore, advances in this technology have demonstrated that double-stranded DNA breaks at sites of vector homology within chromosomal DNA can dramatically increase targeting efficiencies with rAAV (34, 36).

To better understand the mechanism and evaluate the frequency of gene repair with rAAV vectors, we created an enhanced green fluorescent protein (eGFP)-based reporter system by inserting a single-base-pair substitution in the eGFP gene that eliminates fluorescence of the translated GFP (35). AAV vectors containing truncated eGFP cassettes were constructed and used to evaluate efficiencies of gene repair in living cells under nonselective conditions. This same assay was also used to evaluate gene repair by rAAV vectors in transgenic mice that were expressing the mutant eGFP in tibialis muscle and primary fibroblasts. Furthermore, direct cellular injection of rAAV DNA into the nucleus or cytoplasm of 293 target cells was also evaluated. Our results demonstrate that infection with rAAV, but not direct cellular injection of viral DNA, can afford fairly efficient levels of gene repair in 293 cells. However, levels of correction were much less efficient in primary mouse embryonic fibroblasts (PMEFs) and were undetectable following in vivo infection of rAAV in the tibialis muscle of transgenic mice. Interestingly, the enrichment of an S-phase cell population using thymidine led to a slightly higher correction efficiency than for other cell cycle phases. In summary, results from this study demonstrate that certain aspects of intracellular viral genome processing and the cell cycle can affect the efficiency of rAAV-mediated gene repair.

MATERIALS AND METHODS

Mutant eGFP target gene and rAAV vectors. The generation of a single base mutation in eGFP that destroys fluorescence has been previously described (35). In brief, the eGFP gene was mutated by introducing a single base substitution (A → C) in the 66th codon. This process changed amino acid Y66 (TAC) to S66 (TCC) (Fig. 1A and B). This mutation does not change the stability of eGFP, but it does completely eliminate fluorescence of the protein (35). Expression plasmids from both the mutant eGFP [pCUBeGFP(Y66S)] and wild-type eGFP (pCUBeGFP) genes were generated under the control of a chimeric cytomegalovirus enhancer-ubiquitin promoter. These plasmids contained the first intron to the ubiquitin gene. Nonfluorescent truncation mutants of the wild-type eGFP expression construct were generated for cloning into rAAV targeting vectors by PCR from the common BamHI site at the 5′ end of the eGFP gene and at various reverse NotI-containing primers at 3′ sites within the eGFP gene (Fig. 2). The BamHI/NotI PCR products and promoter-poly(A) segments of the transgene cassette were cloned into a rAAV2 proviral plasmid to create various truncation mutants for targeting (Fig. 2). All truncated eGFP targeting vectors were confirmed by DNA sequencing and named according to the base location of truncation in the eGFP gene relative to the starting codon at +1.

rAAV2 was prepared, as previously described, in 293 cells using a three-plasmid transfection system (33, 50). Helper-free virus stocks were treated with nuclease and purified by high-performance liquid chromatography (13, 15, 32). Physical titers of rAAV were determined by slot blot hybridization, and peak fractions were combined for analysis (18, 52). rAAV proviral plasmids pAV2-406, pAV2-516, pAV2-576, pAV2-636, and pAV2-636pA- were used to generate rAAV vectors AV406, AV516, AV576, AV636, and AV636pA-, respectively.

pCUBeGFP(Y66S) expression systems for analysis of gene targeting. The generation of transgenic mice expressing the CUBeGFP(Y66S) gene has been described elsewhere (35). PMEFs were produced from day 13.5 to 15.5 pCUBeGFP(Y66S) transgenic mouse embryos, as previously described (48). Primary fibroblasts were cultured in a high-glucose Dulbecco's modified Eagle's medium and supplemented with glutamine, 10% fetal bovine serum, and 1 mM β-mercaptoethanol. They were then kept in a 5% CO_2 atmosphere at 37°C. The CUBeGFP(Y66S) target gene was introduced into 293 cells by cotransfection of pCUBeGFP(Y66S) and pSV-Neo plasmids, followed by G418 (500 μg/ml) selection. Clones that contained a single copy of the transgene were isolated by screening Southern blot hybridization profiles of genomic DNA from isolated clones against plasmid copy-number controls. This process was performed using an eGFP DNA probe. Expression of the eGFP(Y66S) protein in single-copy 293 cell clones, transgenic PMEFs, and transgenic mouse tibialis muscle was analyzed by Western blotting using a rabbit anti-eGFP antibody (Clontech, Palo Alto, Calif.). The 293 cell line containing a single integrated copy of the eGFP(Y66S) gene with the highest expression of the mutant protein was used in all subsequent experiments. Similarly, the best expressing transgenic line of the eight screened founders was used for all experiments.

Chemical treatments and rAAV gene correction assays. All chemicals were obtained from Sigma-Aldrich (St. Louis, Mo.). Chemical stock solutions were prepared and stored per the manufacturer's instructions. 293 cells expressing the mutated eGFP were treated with various agents known to induce rAAV transduction before rAAV-mediated gene repair was evaluated. These treatments included exposure to *N*-acetyl-L-leucyl-norleucine (LLnL; 10 μM), hydroxyurea (HU; 40 mM), or etoposide (EP; 3 μM) for 16 h prior to rAAV infection. Cells were also irradiated with 5-kJ UV prior to infection. Untreated cells were used as a negative control.

To enrich for cells at different stages of the cell cycle, we applied previously described protocols with the following modifications (7). Briefly, 293 cells were treated with 10 ng of nocodazole/ml overnight to enrich for G_2/M-phase cells. Serum starvation was then employed to enrich for 293 cells in the G_0/G_1 phase of the cell cycle (0.5% fetal bovine serum medium for culture 36 to 48 h). Treatment of 293 cells with 10 μM lovastatin was used to enrich for G_1-phase cells. Treatment with thymidine (2 mM) overnight was used to synchronize 293 cells at the onset of the G_1/S phase of the cell cycle.

The majority of gene repair assays in 293 cells were performed in 96-well plates with 2×10^4 target cells per well (~20% confluence) at the time of viral infection (multiplicity of infection [MOI] = 10,000 particles/cell). Following a 24-h infection, medium was replaced every 24 h until the plates were confluent (3 to 7 days). Targeted correction was observed as eGFP-positive foci under a fluorescence microscope, and each CFU was assumed to be a single gene correction event. The relative correction efficiency was calculated by counting eGFP-positive foci per confluent well (5×10^4 cells) ($n = 4$). Gene repair assays comparing 293 cells and PMEFs were similarly performed in six-well plates seeded with 3×10^5 cells at the time of infection. The relative correction efficiency was then calculated by counting eGFP-positive foci per confluent well (10^6 cells) ($n = 4$).

Clonal analysis of gene correction events and rAAV integration. 293 eGFP(Y66S)-expressing cells were infected with rAAV vectors (MOI = 10,000 particles/cell) in 24-well plates harboring the various truncated eGFP targeting sequences. They were then expanded 5 days after infection until they were 100% confluent in a 100-mm dish (approximately 10^7 cells). EGFP-positive cells were then quantified by fluorescence-activated cell sorting (FACS) analysis and seeded onto 96-well plates at a concentration of one cell per well for clonal expansion. The FACS Vantage SE with FACS Diva option (Becton Dickinson, San Jose, Calif.) was used to perform these studies. Genomic DNA was isolated from clones with functionally corrected eGFP and was amplified using primers EL822 (5′-CCCGGGATCCACCGGTCGCCACCAT3′) and EL823 (5′-GACG CGGCCGCCGTCCTCCTTGAAGTCGATGCCC). The PCR products were sequenced using EL822 primer to confirm the correction of mutated eGFP. Additionally, genomic DNA from 24 independent GFP-positive corrected clones was evaluated by Southern blotting to determine the extent of AAV integration and potential rearrangements at the target GFP locus.

Isolation of rAAV DNA and single-cell injections. High-performance liquid chromatography-purified AV636 vector stock was digested with proteinase K digestion buffer (50 mM Tris-Cl, 10 μg of proteinase K/ml, 50 mM EDTA, 0.1% sodium dodecyl sulfate; pH 8.0) at 37°C overnight, followed by two sequential extractions with phenol, phenol-chloroform, and chloroform. Viral DNA was then ethanol precipitated and dissolved in Tris-EDTA buffer. Viral DNA concentrations were estimated by Southern blotting against plasmid control standards, and the DNA was evaluated by both nondenaturing and NaOH-denaturing agarose gels, as previously described (14). Single-cell microinjection experiments were essentially performed as previously described (35). However, cytoplasmic and nuclear injections were both evaluated separately. Both heat-denatured (85°C for 15 min followed by quenching on ice) and nondenatured viral DNA was evaluated in these experiments, in which approximately 5,000 vector genomes were injected per cell. In total, ~2,500 mutant eGFP 293 target

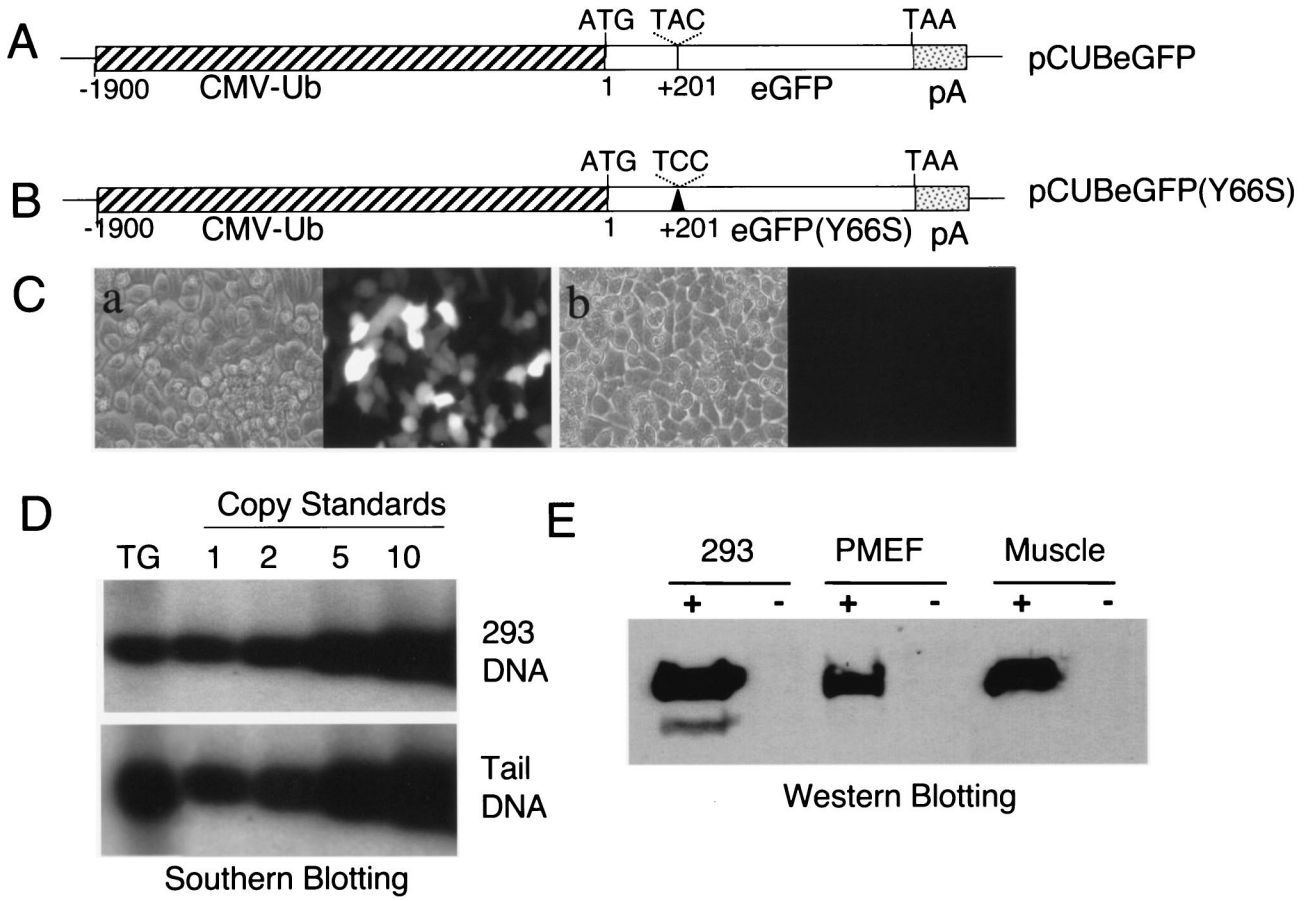

FIG. 1. Generation of cell lines expressing a mutant eGFP gene. (A) Schematic of the wild-type eGFP transgene cassette (pCUBeGFP), including a cytomegalovirus enhancer and a human ubiquitin promoter. The starting code ATG in eGFP is designated as +1. (B) A single-base A→C substitution was introduced at bp 201 of eGFP, resulting in a 66Tyr-to-66Ser amino acid conversion and the generation of pCUBeGFP (Y66S). (C) Transfection of HeLa cells with pCUBeGFP (a) or pCUBeGFP(Y66S) (b). Nomarski and fluorescent images are given to the left and right of each panel, respectively. (D) Southern blotting was used to determine the copy number of pCUBeGFP(Y66S) transgenes (TG) in genomic DNA from a 293 cell clone and a tail DNA from a transgenic line. The genomic DNA was digested with XbaI and NotI and probed with a ^{32}P-labeled eGFP(Y66S) cDNA probe. Copy number standards are given at the top of the blot. (E) Western blotting with an anti-GFP antibody was used to determine the extent to which pCUBeGFP(Y66S) transgenes were expressed in the 293 target cell clone, in PMEFs, and in tibialis muscle from the transgenic line. Transgene-positive samples are marked by a +, and negative controls lacking the transgene are marked by a −.

cells and ~1,400 PMEFs from the transgenic mouse model were evaluated by direct nuclear or cytoplasmic injection of both forms of viral DNA.

Evaluation of rAAV-mediated gene correction in transgenic mice. To evaluate the efficiency of rAAV-mediated gene repair in vivo, the tibialis muscles of CUBeGFP(Y66S) transgenic mice were injected with 10^{10} particles of the AV636 vector/muscle. Injected muscles were harvested and evaluated for GFP fluorescence en face in fixed cryoprotected sections at 4, 8, and 15 weeks postinfection, as previously described (16).

RESULTS

Generation of targeting models expressing the mutant eGFP(Y66S) protein. In an effort to develop a simple model system for detecting a single base-pair conversion in vitro and in vivo, our investigators have previously described a functional eGFP reporter system capable of evaluating gene correction events in live cells and in the absence of selection (35). For the purpose of the present study, we developed and characterized both in vivo transgenic and in vitro cell line models that effectively express the mutant eGFP(Y66S) protein in 293 cells, PMEFs, and mouse tibialis muscle (Fig. 1E). The 293 cell model was specifically developed to have a single-copy target gene, while PMEFs and tibialis muscles of the transgenic line contained approximately five copies of the mutant eGFP(Y66S) target (Fig. 1D).

Gene correction with rAAV vectors containing homology to a mutant eGFP target. Five rAAV vectors were evaluated for their ability to mediate gene repair of the integrated mutant eGFP target gene in 293 cells. All vectors contained 1.9 kb of homology upstream of the mutation, as well as different lengths (ranging from 205 to 435 bp) of downstream homology (Fig. 2). Additionally, a construct with poly(A) deleted with 435 bp of short-end homology (AV636pA-) was also prepared to evaluate whether extended noncontinuous homology to the target would increase the efficiency of gene targeting (Fig. 2). It was confirmed that each of the rAAV vectors used for eGFP gene correction did not produce fluorescent eGFP by proviral plasmid transfection and infection with the viral vectors (data

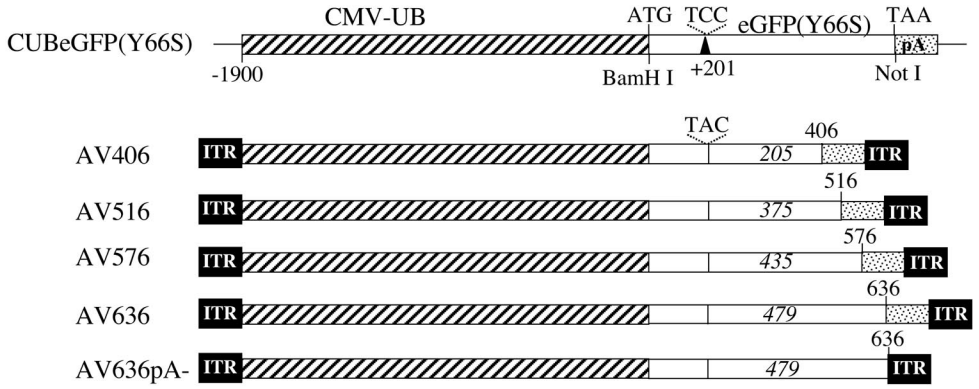

FIG. 2. rAAV gene targeting vectors. rAAV2-based vectors were generated to have the indicated areas of homology to the mutant eGFP target (top). Areas of similar shading represent homology between vectors and the mutant target. The numbers in italics give the length of short-end homology from the mutant base. All vectors have a common long-arm homology of 2,100 bp. The names of various rAAV vectors reflect the positioning of the truncation as base pairs relative to the starting codon of the GFP gene (ATG) at bp +1.

not shown). Attempts to generate constructs with longer short-end homology (i.e., pAV680) resulted in partial fluorescence of eGFP in transfected cells and, therefore, were not pursued. Functional restoration of eGFP fluorescence was seen with all five rAAV vectors analyzed (AV406, AV516, AV576, AV636, and AV636pA-), with a trend toward higher levels of correction in vectors with longer short-end homology to the target (Fig. 3A).

Several general correlations between the efficiency of correction and the structure of the vector genome were observed (Fig. 3). First, increases in short-end homology from 205 to 375 bp significantly increased targeting efficiencies ($P < 0.05$), while extending the short-end homology from 375 to 435 bp did not significantly affect targeting. Unfortunately, we were unable to extend homology further to test whether a similar upper limit in homology exists, since longer ends of homology reconstituted eGFP fluorescence. Second, we observed no significant difference in targeting efficiency between AV636 and AV636pA- vectors, suggesting that extended homology to the target, if noncontinuous with the mutation, does not increase gene correction. These findings support the current hypothesis (25, 26) that correction by rAAV is mediated by homologous recombination, not by gene repair. Time course studies also demonstrated that maximal levels of correction were achieved by 5 days postinfection of 293 cells (Fig. 3B). Although rAAV-mediated correction was also observed following infection of PMEFs from the CUBeGFP(Y66S) transgenic line (Fig. 3E), only 6 to 13 eGFP-positive foci were observed from a target pool of 10^6 cells at 10 days following infection with AV636. This level of correction observed was 50- to 100-fold less efficient than that seen in 293 cells (Fig. 3C). Importantly, the level of rAAV gene transduction in PMEFs was 20- to 30-fold less efficient than that in 293 cells following infection with a rAAV2 luciferase vector (data not shown).

We hypothesized that the lower level of rAAV-mediated gene repair in PMEFs might be related to previous findings that intracellular blocks in rAAV transduction exist in fibroblasts, resulting in poor gene transduction efficiencies (23, 38). Genotoxic stress and proteasome inhibitors have been studied as agents that enhance gene transduction of rAAV in vitro and in vivo by enhancing either nuclear accumulation of the virus or enhancing second-strand synthesis of its genome (1, 2, 17, 41, 44–46). Consequently, we sought to test whether augmentation of rAAV transduction (i.e., expression of an expressed transgene) would also enhance rAAV-mediated gene repair. Several agents, including LLnL, UV irradiation, HU, and EP, were tested. Each of these agents enhanced rAAV transduction in both 293 cells and in PMEFs when AV2.Luciferase vectors were used for infection (Fig. 4; data not shown for PMEFs). However, no enhancement in gene repair rates was observed with any of the treatments (Fig. 4; data not shown for PMEFs). These findings support previous observations that there is no direct correlation between gene targeting and transduction with rAAV vectors following genotoxic stress (26). However, we were surprised to find that LLnL, which is thought to enhance transduction of rAAV vectors by increasing nuclear accumulation of the virus, did not enhance targeting. These findings suggest that fundamentally different mechanisms may be involved in transduction and gene correction with rAAV.

Molecular confirmation of gene correction in 293 cells. To more quantitatively evaluate the efficiency of gene repair and to confirm the molecular correction of the mutant eGFP gene in chromosomal DNA, AV636-infected 293 CUBeGFP(Y66S) cells were analyzed by FACS analysis at 7 days postinfection, and fluorescent cells were separated individually for clonal expansion in 96-well plates. The data from the FACS analysis revealed that roughly 0.1% of the total cell population expressed fluorescent eGFP. Clonal expansion of fluorescent cells and PCR sequencing of the eGFP gene demonstrated the expected single-base conversion at position 201 of the eGFP coding region from C to T without any other base alteration (Fig. 5). Furthermore, this genetic correction was stable during clonal expansion.

To test the efficiency of the genomic integration of rAAV genomes and evaluate any potential target site rearrangements in corrected cells, we randomly selected 24 of the corrected pCUBeGFP(Y66S) 293 cell clones and performed Southern blotting analysis using a truncated eGFP-poly(A) probe. One of the 24 clones gave an extra transgene band in addition to the endogenous target GFP locus of the pCUBeGFP(Y66S) 293 cell line (Fig. 6A, clone 13). This extra band was indicative of an integration event at the nontarget locus. Additionally, the

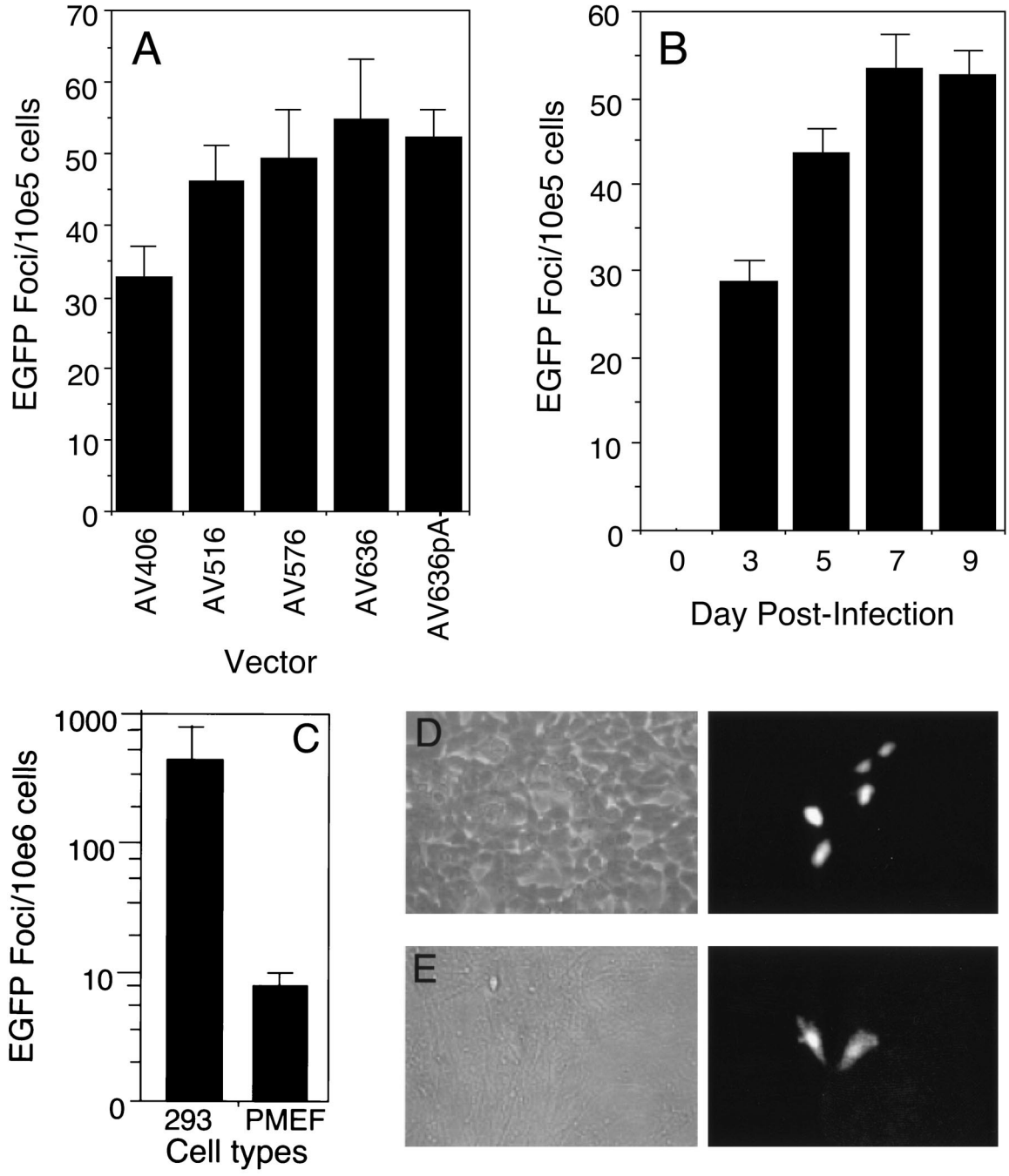

FIG. 3. Influence of short-end homology and time on gene correction. (A) Comparison of various rAAV vector constructs to correct a mutant GFP target in pCUBeGFP(Y66S) 293 cells. Cells were evaluated for GFP-positive CFU in 96-well plates, as described in Materials and Methods, at 7 days postinfection with 10,000 particles/cell. (B) Time course of gene correction following AV636 infection of pCUBeGFP(Y66S) 293 cells at 10,000 particles/cell. (C) Comparison of gene repair efficiencies in 293 cells and PMEFs harboring the pCUBeGFP(Y66S) target gene. Cells were evaluated for GFP-positive CFU in six-well plates, as described in Materials and Methods, at 9 days postinfection with 10,000 particles/cell. Results in panels A, B, and C present the mean (± standard error of the mean) EGFP foci per indicated number of cells for four independent experiments. Cell numbers were derived at the time of CFU analysis (confluency) and not at the time of infection. (D) Representative fluorescent photomicrograph of a corrected CFU in pCUBeGFP(Y66S) 293 cells. (E) Representative fluorescent photomicrograph of a corrected CFU in pCUBeGFP(Y66S) PMEFs. Phase-contrast images are given to the left of the fluorescent portion in panels C and D.

target GFP locus demonstrated no detectable rearrangements in any of the 24 corrected clones. These data suggest that under the conditions of our studies, the frequency of random integration (1 out of 24 clones, or 4%) is substantially higher than the frequency of gene correction events (quantified by FACS analysis as 0.1%).

Effects of the cell cycle phase on gene targeting with rAAV. It has been suggested that rAAV vector transduction occurs

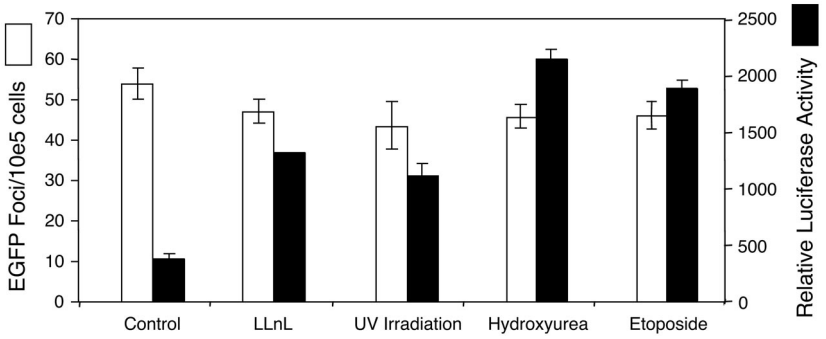

FIG. 4. Correlation of rAAV transduction efficiencies with gene repair. pCUBeGFP(Y66S) 293 cells were treated with LLnL (10 μM), UV irradiation (5 kJ/m^2), HU (40 mM), or EP (3 μM) immediately prior to infection with an AV636 or AVLuc virus at an MOI of 10,000 particles/cell. At 7 days postinfection, cells were harvested for luciferase activity or quantified for GFP-positive CFU. Results depict the mean ± standard error of the mean for four independent experiments. The various compounds also similarly induced AVLuc gene expression in PMEFs, but no changes were observed in gene correction following infection with AV636 (data not shown).

more efficiently in cells that have entered the S phase of the cell cycle (41, 44). To determine whether the phase of the cell cycle might also affect the efficiency of gene repair with rAAV, we evaluated gene targeting in 293 cell populations enriched for G_0/G_1, G_1, G_1/S, and G_2/M phases of the cell cycle. Enrichment of cells at the G_0/G_1 phase was induced by serum starvation, while lovastatin inhibited 3-hydroxy-3-methyglutaryl-coenzyme A reductase, resulting in the enrichment of

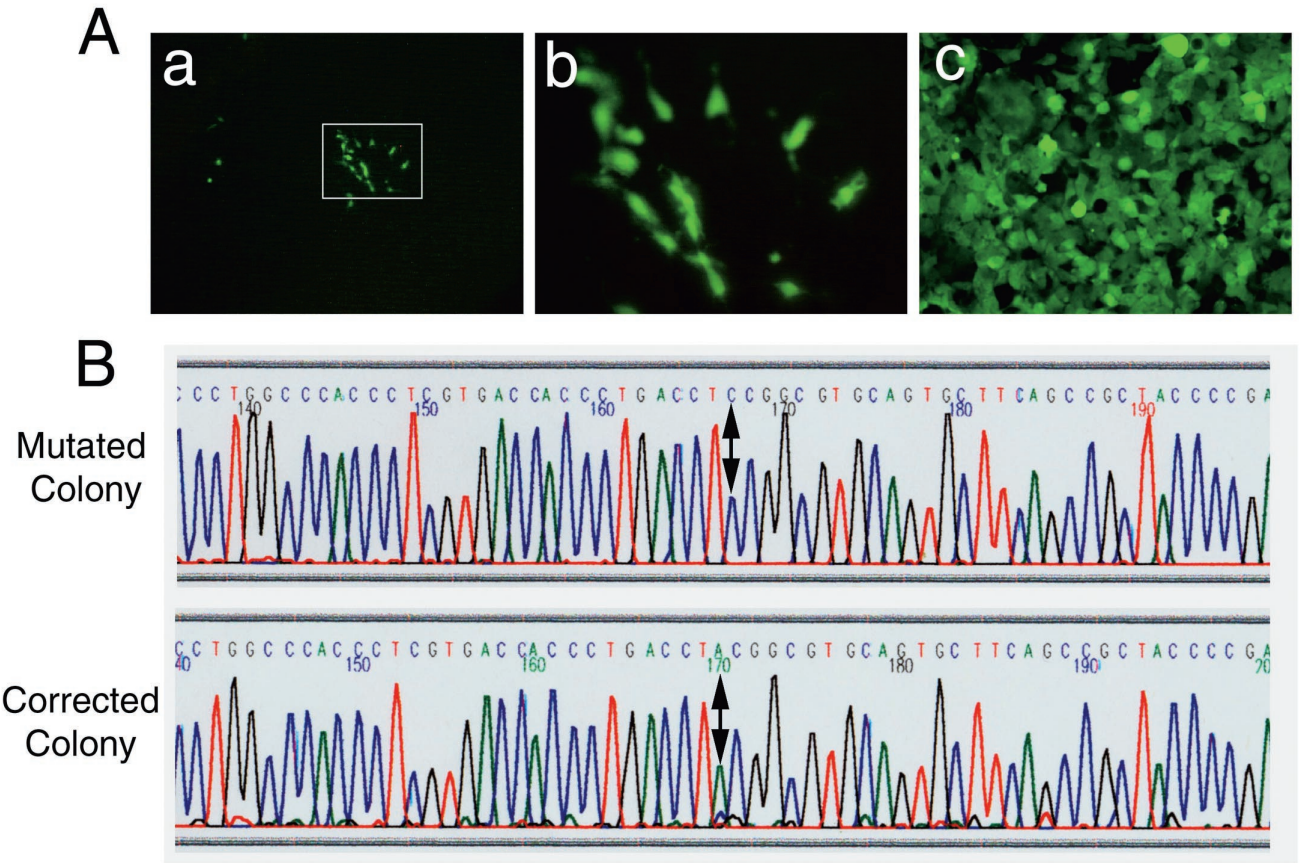

FIG. 5. Confirmation of permanent site-specific gene correction in eGFP-targeted 293 cells. (A) pCUBeGFP(Y66S) 293 cells were infected with AV636 at an MOI of 10,000 particles/cell and expanded in a 100-mm dish prior to FACS analysis. Corrected clones are shown in a presorted population (a), and the boxed region is enlarged in panel b. Following AV636 infection and FACS, 0.1% of cells scored positive for GFP. Single cells that expressed GFP were expanded into 96-well plates (c). A representative GFP-positive expanded clone is shown. Approximately 5% of single-cell clones did not demonstrate eGFP fluorescence. (B) Genomic DNA was isolated from five fluorescent and nonfluorescent expanded clones, and the GFP target sequence was PCR amplified and sequenced. The sequences from all nonfluorescent 293 expanded clones contained the single-base-pair mutation at nucleotide 201 (top panel). However, sequences from the fluorescent colonies demonstrated the site-specific C-to-A conversion at this position (bottom panel).

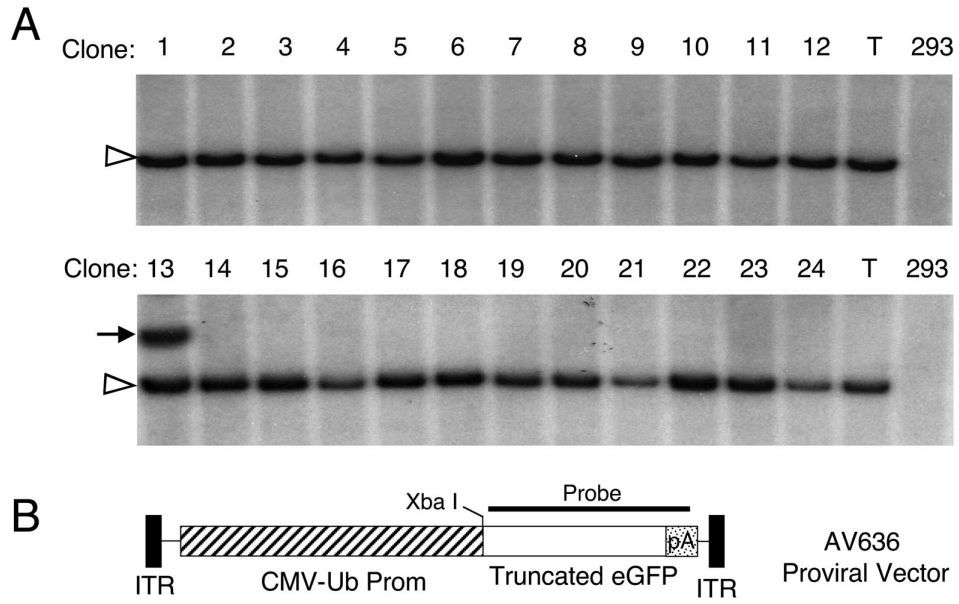

FIG. 6. Southern blot analysis of rAAV integration events and eGFP target locus rearrangements in corrected 293 cell clones. pCUBeGFP(Y66S) 293 cells were infected with AV636 at an MOI of 10,000 particles/cell, and FACS sorting was used to isolate single cells which expressed GFP. A total of 24 clones were expanded, and genomic DNA from these clones was isolated for Southern blot analysis. All clones retained stable GFP expression after isolation. Genomic DNA was digested with XbaI and Southern blots were hybridized to a ^{32}P-labeled truncated eGFP(Y66S) poly(A) probe. (A) Southern blotting analyzing AV636-corrected pCUBeGFP(Y66S) 293 cell clones. Only clone 13 gave an extra banding (solid arrow) that is indicative of an AV636 integration event at the nontarget locus. All clones showed no detectable alterations in the endogenous pCUBeGFP(Y66S) target transgene (open arrowhead). T, pCUBeGFP(Y66S) 293 cells without AAV infection; 293, 293 cells without pCUBeGFP(Y66S) transgene or AAV infection. (B) The AV636 proviral vector and placement of the XbaI restriction site and probe used for Southern analysis. The single band seen with pCUBeGFP(Y66S) 293 cell DNA (lane T) is consistent with a single-copy transgene as determined in Fig. 1D.

cells at the G_1 phase. Since microtubule formation is an important structural feature of cells as they enter mitosis, we used nocodazole, which chemically interferes with the organization of microtubules in cells, to enrich the G_2/M phase (7). Synchronization of cells at the onset of the G_1/S phase is generally achieved by using chemical agents to inhibit DNA synthesis, such as aphidicolin, HU, or excess thymidine (7, 41, 44). Our data confirmed previous results that HU treatment has no effect on gene targeting by AAV vectors (Fig. 4) (26). Thus, we used a double-thymidine block to enrich cells at the G_1/S-phase border (7). Since some of these agents also affect rAAV transduction, we removed all compounds after the initial infection period and evaluated gene correction at 3 to 7 days postinfection. Our results indicated that the rate of gene targeting induced during the S phase (thymidine induced) was ~2-folder higher than in other cell phases (Fig. 7). These findings suggest that cells at the G_1/S phase of the cell cycle may be most receptive to gene targeting with rAAV.

Intercellular rAAV genome processing plays an important role in gene repair. Intercellular trafficking of AAV has been shown to play a critical role in rAAV gene transduction in both AAV-permissive and -nonpermissive cells (19, 23, 24). It has also been suggested that processing of AAV capsids by ubiquitination is important in viral trafficking to the nucleus and in the potential uncoating of the virion (51). Although it is currently unclear if rAAV uncoats in the cytoplasm or within the nucleus, evidence to support both claims is present in the literature.

The elaborate nature of intracellular processing of AAV virions prompted us to address whether processing of the rAAV genomic DNA may also be necessary for gene repair events with this virus. To indirectly test whether intracellular processing of the rAAV viral genome is needed to prime the genome to carry out gene repair events, we asked whether direct cellular injection of purified rAAV viral DNA could facilitate gene repair in our 293 mutant eGFP model. To this end, purified AV636 viral DNA was directly microinjected into either the cytoplasm or the nucleus of targeted cells, and cells were monitored for GFP fluorescence over the course of 15 days. This method of administration allows a cell to receive ~5,000 copies of AAV genome when 1.0 μg of viral DNA/μl is injected. Molecular evaluation of the purified viral DNA demonstrated extreme heterogeneity in the apparent molecular weight of genomes in nondenaturing agarose gels, suggesting that a large portion of DNA likely annealed during preparation to yield double-stranded and concatamerized genomes (Fig. 8A). Hence, we also adopted a method of heat denaturing DNA in order to form more uniform monomer genomes. Heat denaturing appeared to promote the formation of ITR-annealed intramolecular base-paired stem-loops when migration was compared on nondenaturing and NaOH denaturing gels (Fig. 8B). Results from direct nuclear injection with both nondenatured and heat-denatured viral DNA failed to give rise to gene repair events in 293 cells (data not shown). Considering that viral DNA may be modified in the cytoplasm prior to nuclear uptake, we also performed studies with cytoplasmic injection of viral DNA. However, again, no gene correction was observed. These failures in rAAV-DNA-facilitated gene

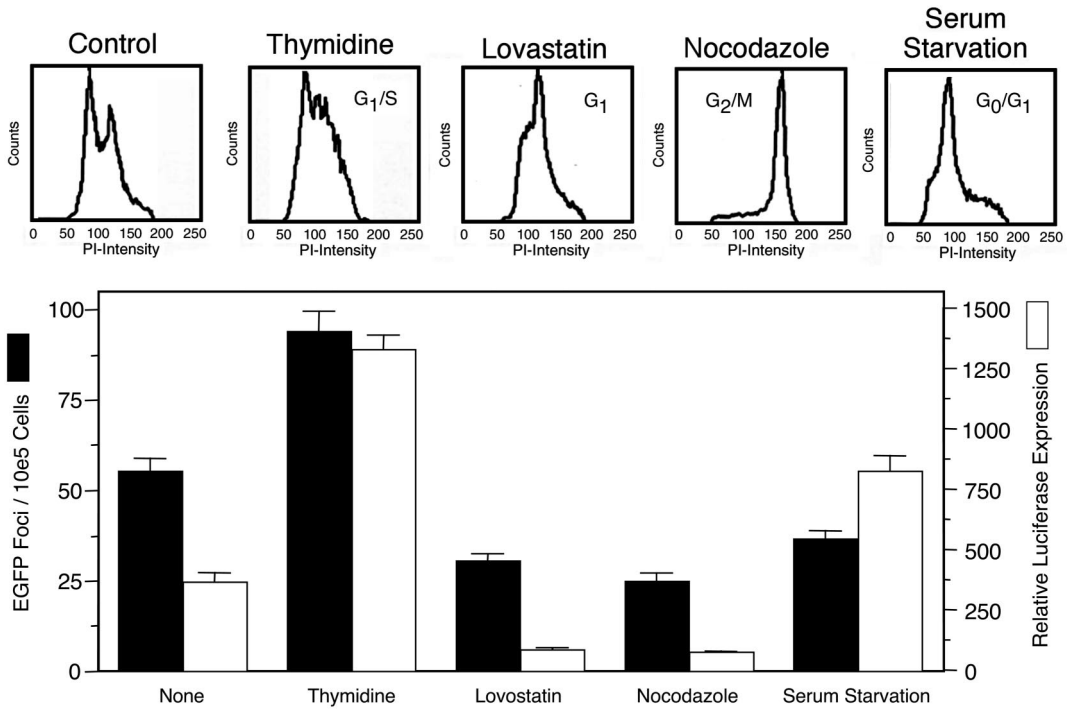

FIG. 7. Effect of cell cycle phase on gene targeting. pCUBeGFP(Y66S) 293 cells were treated with the indicated chemical compounds, as described in Materials and Methods, to enrich for specific phases of the cell cycle. Cells were then infected with AV636 at an MOI of 10,000 particles/cell and evaluated for gene repair events by quantifying eGFP-positive CFU at 7 days postinfection. Similarly, separate plates of cells were infected with AVLuc at an MOI of 1,000 particles/cell to assess for changes in transduction efficiency. The top panel indicates chemically induced changes in the cell cycle based on propidium iodide FACS analysis. The bottom panel depicts results quantifying changes in both GFP gene repair events and luciferase expression induced by the various chemical treatments. Results depict the mean ± standard error of the mean for four independent experiments.

repair were seen despite successful expression of a virally expressed wild-type GFP transgene following cytoplasmic and nuclear injection of rAAV DNA (Fig. 8C). Compared to injections with nondenatured viral genomes that contain double-stranded molecules, the expression of eGFP from denatured viral genomes lagged by 1 to 2 days postinjection, as might be expected since these single-stranded genomes would have to replicate a second strand prior to expression of the transgene.

rAAV-mediated gene correction in mouse tibialis muscle. To study rAAV gene targeting in vivo, we also injected the AV636 vector into the tibialis muscles of CUBeGFP(Y66S)-expressing transgenic mice. Although the mutant eGFP was efficiently expressed in this mouse line in tibialis muscle (Fig. 1), no functional eGFP fluorescence was observed up to 15 weeks postinfection (data not shown). At the dose of rAAV used in this study, approximately 85% of the muscle fibers can be transduced by 15 weeks, suggesting that the efficiency of gene targeting is quite low in this tissue.

DISCUSSION

rAAV continues to be one of the most diverse and powerful gene transfer vector systems currently in the gene therapist's armamentarium. Within an increasing number of serotypes being identified from multiple animal species, rAAV vectors are now able to infect most organs and cell types with high efficiency. rAAV has also been extensively studied as a gene targeting agent, with capabilities of correcting genomic alterations through homologous recombination with vector-derived sequences. Work in this area has primarily been pioneered by Russell and colleagues (25, 26, 29, 30, 34, 42, 43), with only one recent report confirming the use of rAAV for gene correction by a second laboratory (36). Although it is currently unclear how rAAV mediates gene repair events, it is assumed that some unique aspect of the single-stranded DNA genome and palindromic ITRs likely play roles in this process. Furthermore, it appears evident that cellular mechanisms of DNA repair are involved in rAAV-mediated gene targeting processes, because double-stranded DNA breaks can increase gene correction rates at sites of vector homology in the genome (34, 36). In this report, we have described a nonselective eGFP-based reporter system that allows for the evaluation of functional gene correction in living cells under nonselective conditions. We have utilized this functional read for gene repair as a method for assessing both the efficiency and mechanism of rAAV gene targeting in vivo and in vitro.

Establishment of the eGFP-based recovery assay in transformed 293 cells demonstrated a ~0.1% gene repair efficiency. This rate was approximately an order of magnitude lower than those achieved when using neomycin and hypoxanthine phosphoribosyltransferase selection systems (30, 42), but it was similar to those achieved with nonselective alkaline phosphatase systems (26, 29). However, unlike previous studies using primary human fibroblasts that had retrovirally integrated al-

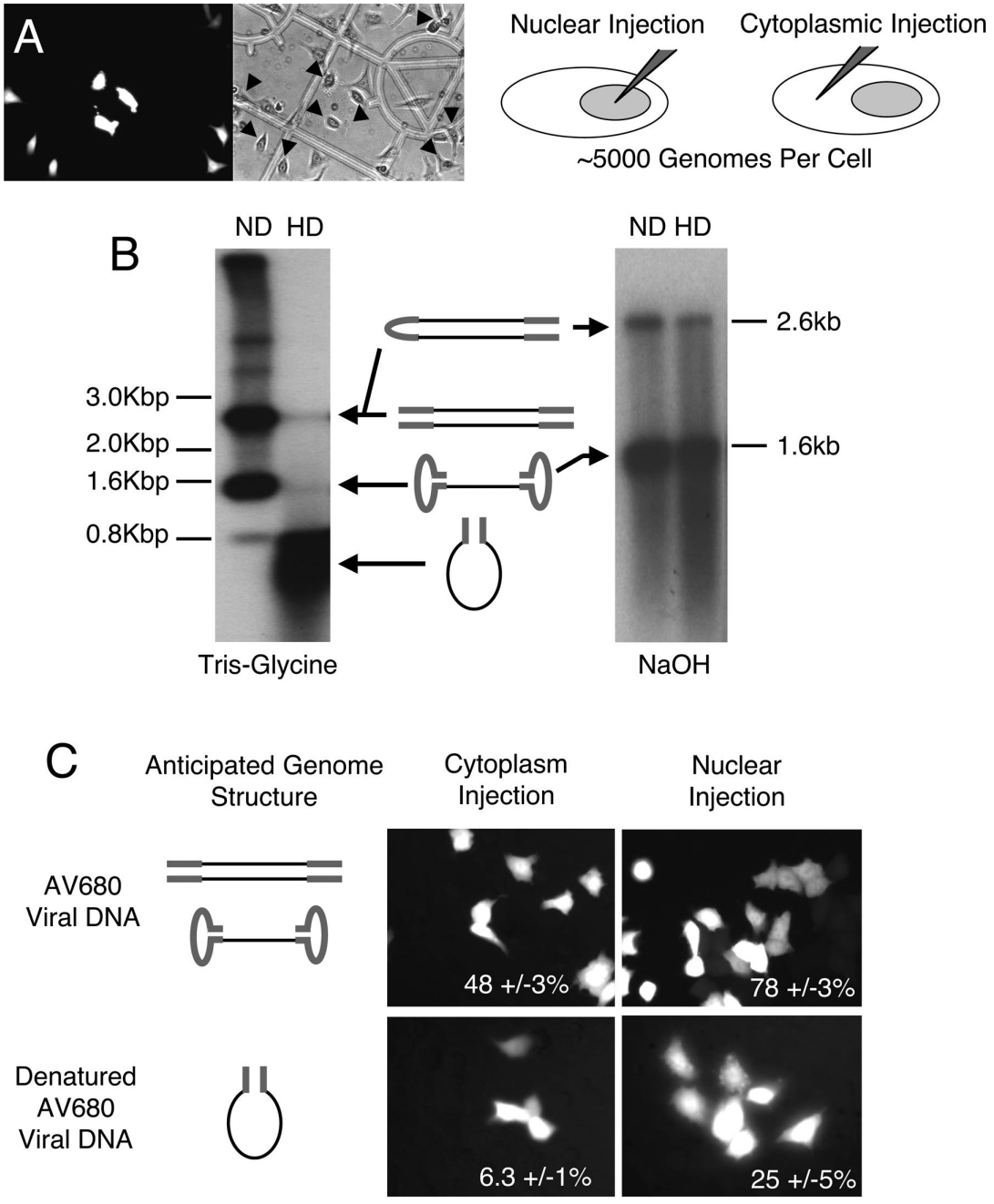

FIG. 8. Single-cell injection studies with rAAV DNA. Direct nuclear or cytoplasmic injection of AV636 viral DNA was evaluated for its ability to correct eGFP mutations in both pCUBeGFP(Y66S) 293 cells and PMEFs. (A) Direct cytoplasmic or nuclear injection of a GFP plasmid expression construct demonstrated approximately 85% injection efficiency (i.e., GFP fluorescence) in 293 cells. The photomicrograph depicts the grids used to track expression in injected cells (arrows). (B) Isolated viral DNA was evaluated for secondary structure using Tris-glycine or NaOH-agarose gels, followed by Southern blotting against a GFP probe. The predicted secondary structure of the viral genomes is shown for both nondenatured (ND) and heat-denatured (HD) viral DNA samples. For heat denaturing, samples were heated at 85°C for 15 min prior to loading. (C) As positive controls, viral DNA isolated from AVeGFP (expressing an intact wild-type GFP gene) was directly injected into the nucleus or cytoplasm in its ND and HD form. GFP expression was assessed at 3 days postinfection and quantified as the percentage of injected cells (in total, 1,200 cells were injected, i.e., three experiments each with 400 cells). Similar injections were performed with pCUBeGFP(Y66S) 293 cells (~2,500 cells per construct) and PMEFs (~1,400 cells per construct) using AV636 viral DNA; however, no GFP fluorescence was observed (data not shown).

kaline phosphatase target sequences (26), our results in PMEFs demonstrated 50- to 100-fold-lower targeting efficiencies than those seen in 293 cells or primary human fibroblasts (26). Although the length of short-end homology was restricted in our present study due to GFP target size constraints, this does not appear to be solely responsible for the lower targeting rates based on the results of previous studies (26). A more likely explanation for lower targeting efficiencies in transgenic

mouse fibroblasts than in human fibroblasts is a lower level of infection due to reduced receptor abundance. Indeed, studies by our laboratory have demonstrated that infection of PMEFs with a rAAV2 luciferase vector gave rise to 10-fold-lower levels of transgene expression than that in the human fibroblasts used by D. W. Russell's laboratory (data not shown). Previous studies have also utilized a retrovirus to establish a complex pool of integration sites, while our present transgenic model has a single integration site. Although our integrated eGFP target was highly expressed in PMEFs, we cannot rule out that this locus has a reduced targeting efficiency due to some inherent genome configuration. Nonetheless, our results demonstrate that targeting in PMEF cells with rAAV2 appears to be much less efficient than in 293 cells and human fibroblasts.

Other similarities between the present study and previous work include the finding that there is no direct correlation between the efficiency of vector transduction (as assessed by transgene expression) and gene targeting. These findings imply that forms of the rAAV genome that facilitate gene repair are likely not double-stranded vector genome conversion products capable of expressing an expressed transgene. Based on these results, we hypothesized that direct cellular injection of purified single-stranded viral DNA might be capable of improving gene repair efficiencies. The present eGFP recovery system provided the ideal venue to assess functional gene correction events at the single-cell level using viral DNA injection. Studies assessing direct nuclear or cytoplasmic injection of purified viral DNA from a functionally intact eGFP vector demonstrated efficient expression of fluorescent eGFP. However, injection of double-stranded or single-stranded vector genomes into mutant eGFP target cells failed to yield any gene correction events. Given the fact that vector genome concentrations within cells following direct injection were at least 100-fold higher than those achieved by infection, we concluded that some feature of vector genome processing is required to prime viral genomes for gene repair. Interestingly, this competency was not achieved by cytoplasmic injection of viral DNA, suggesting that some aspect of viral genome processing that involves uncoating may be required. Alternatively, it is possible that purification of viral DNA removed some cofactors, such as Rep, which is covalently linked to the viral DNA. Indeed, some reports have suggested that Rep may be contained within AAV virions (37).

Although cell cycling is not essential for rAAV transduction, S-phase cells are significantly more receptive to productive infection with rAAV. One explanation for this finding is that cellular DNA polymerases active in the S phase convert single-stranded AAV genomes to transcriptionally active double-stranded molecules (41, 44). Recent studies have also revealed that the gene mismatch repair system is required for S-phase checkpoint activation (8). Our results demonstrated that thymidine-induced G_1/S block enhances rAAV gene targeting ~2-fold in 293 cells, suggesting that this phase of the cell cycle may contain a higher abundance of the cofactors required for gene targeting than other phases of the cell cycle. However, DNA synthesis inhibitors that also arrested cells at the S phase had no effect on AAV gene targeting. Furthermore, in all other phases of the cell cycle, a decrease in gene targeting was observed. Hence, the augmentation seen in rAAV gene targeting in the presence of thymidine may be independent of the cell cycle and could instead be a more direct reflection of the gene repair status of cells.

Given the lack of correlation between the efficiency of gene expression from a rAAV vector and the efficiency of gene correction, we sought to utilize rAAV2 as the serotype for testing gene correction in vivo. In tibialis muscle, the onset of gene expression is quite slow for rAAV2, so we reasoned that the slower conversion of vector genomes to circular forms might enhance the rate of gene correction. However, our findings of no functional correction in the mouse model following infection of the tibialis muscle substantiate the lower rates of gene correction in PMEFs. The inefficiency of rAAV to facilitate gene correction in muscle could be a feature of that tissue type (i.e., differentiated and noncycling) or could be limited by the effective MOI. Additionally, muscle has been shown to efficiently convert rAAV genomes to circular molecules, and these forms may not be the intermediates of rAAV that facilitate gene repair. Although no in vivo demonstration of rAAV repair has yet been reported, other tissue targets are certainly worth investigating.

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